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Constitutive overexpression of Cu/Zn superoxide dismutase exacerbates kainic acid-induced apoptosis of transgenic-Cu/Zn superoxide dismutase neurons

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ABSTRACT Cu/Zn superoxide dismutase (Cu/Zn SOD) is a key enzyme in the metabolism of oxygen free radicals. The gene resides on chromosome 21 and is overexpressed in patients with Down syndrome. Cultured neurons of transgenic Cu/Zn SOD (Tg-Cu/Zn SOD) mice with elevated activity of Cu/Zn SOD were used to determine whether constitutive overexpression of Cu/Zn SOD creates an indigenous oxidative stress that predisposes the Tg-Cu/Zn SOD neurons to added insults. Neurons from three independently derived Tg-Cu/Zn SOD strains showed higher susceptibility than nontransgenic neurons to kainic acid (KA)-mediated excitotoxicity, reflected by an earlier onset and enhanced apoptotic cell death. This higher susceptibility of transgenic neurons to KA-mediated apoptosis was associated with a chronic prooxidant state that was manifested by reduced levels of cellular glutathione and altered $[Ca^{2+}]_i$ homeostasis. The data are compatible with the thesis that overexpression of Cu/Zn SOD creates chronic oxidative stress in the transgenic neurons, which exacerbates their susceptibility to additional insults such as KA-mediated excitotoxicity.

A growing body of evidence implicates oxygen free radicals (OFR) in a broad range of neuropathologies including Parkinson disease (for review, see refs. 1 and 2), Alzheimer disease (AD) (for review, see refs. 1, 3, and 4), and amyotrophic lateral sclerosis (ALS) (for review, see refs. 5 and 6), as well as in the induction of apoptosis (7, 8). Under normal physiological conditions, there is an equilibrium between oxidant and antioxidant mechanisms (3, 4); when this balance is upset, OFR formation is favored, leading to oxidative stress and cell damage. The enzyme Cu/Zn superoxide dismutase (Cu/Zn SOD), which catalyzes the conversion of superoxide radicals ($O_2^{\cdot}$) into hydrogen peroxides (H_2O_2), plays an important role in the metabolism of OFR (for review, see ref. 9). When activity of Cu/Zn SOD increases, H_2O_2 accumulates and its reaction with transition metals (Fenton's reaction) is facilitated (3, 10). This may even occur when H_2O_2 comes into contact with the reduced form of copper (Cu^+) in Cu/Zn SOD. The product of Fenton's reaction is the hydroxyl radical (•OH), the most reactive and noxious OFR species. Aside from the legitimate dismutation reaction, Cu/Zn SOD is able to catalyze surrogate reactions such as the production of •OH using anionic scavengers and H_2O_2 (10, 11) and nitration of protein tyrosine residues by peroxynitrite (12). Several reports by us (13–18) and by others (19–22) showed that an increase in Cu/Zn SOD activity may cause oxidative damage. This may be due to the fact that Cu/Zn SOD functions as a component in a pathway and alterations in the amount of enzyme disrupt the entire chain or it may be because increased enzyme production promotes the side reactions mentioned above. In Down syndrome (DS), the phenotypic manifestation of trisomy 21, the activity of Cu/Zn SOD is elevated because the gene for Cu/Zn SOD resides on chromosome 21. DS patients suffer from a wide range of symptoms and they usually develop AD pathology during the fourth decade of their lives (for review, see ref. 23). Significantly, increased generation of OFR and enhanced apoptosis were recently found in cultured DS neurons (24), suggesting that DS neurons have a defect in the metabolism of reactive oxygen species that may contribute to the mental retardation and early development of AD (24).

We previously investigated the possible involvement of Cu/Zn SOD overproduction in cell damage through the use of cellular and animal model systems. We found that stably transfected cells overexpressing Cu/Zn SOD exhibited substantially increased lipid peroxidation (13) and had a specific lesion that affects the chromaffin granule's proton pump, which plays an important role in the uptake of neurotransmitters into the vesicles (14). In the transgenic-Cu/Zn SOD (Tg-Cu/Zn SOD) mice, a similar defect was also identified in an analogous organelle, the platelet's dense granule, which is responsible for the uptake and storage of blood serotonin (17). It was also found that Tg-Cu/Zn SOD mice have certain thymus and bone marrow abnormalities that resemble the thymic defects in children with DS (18). These findings suggested that overexpression of Cu/Zn SOD in transgenic mice can indeed cause certain physiological abnormalities similar to those found in DS. These findings also indicated that defects caused by increased Cu/Zn SOD can have a highly specific subcellular target, and thus serve as a paradigm f r how alterations in Cu/Zn SOD expression could cause oxidative stress mediated cell injury leading to neurodegenerative disease like ALS (for review, see ref. 25). In this context, it is important to note that the neuromuscular juncti ns of Tg-Cu/Zn SOD mice display significant abnormalities and morphological changes including withdrawal and destruction of some terminal axons and development of multiple small terminals (15, 16, 26). Some of these subclinical changes are reminiscent of the pathology observed in transgenic mice with ALS-like disease, which was generated by overexpression of a mutated Cu/Zn SOD gene bearing familial ALS mutations (see ref. 25 and references therein).

In the present study, we used cultured neurons from Tg-Cu/Zn SOD mice to investigate the relation between increased expression of Cu/Zn SOD and kainic acid (KA)-induced exitotoxicity, with the aim of gaining insight into the role of altered Cu/Zn SOD expression and oxidative stress in neuronal death.

MATERIALS AND METHODS

Tg-Cu/Zn SOD Mice and Primary Brain Cultures. Three independently produced strains of Tg-Cu/Zn SOD mice

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Abbreviations: OFR, oxygen free radicals; Cu/Zn SOD, Cu/Zn superoxide dismutase; Tg-Cu/Zn SOD, transgenic-Cu/Zn SOD; KA, kainic acid; ALS, amyotrophic lateral sclerosis; DS, Down syndrome; AD, Alzheimer disease; GSH, glutathione; mBcl, monochlorobimane; NMDA, *N*-methyl-D-aspartate.

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TgHS 51, TgHS 69, and TgHS 70) carrying four to five copies of the human Cu/Zn SOD gene (17, 27) and control nontransgenic mice were used. Tg-Cu/Zn SOD male and female mice homozygous for the transgene were bred and used for timed pregnancies in the present experiments.

Scheduled matings were performed, pregnant mice were killed, and 14-day-old fetuses were removed by cesarean section. Brains were removed and tissue was mechanically dispersed by triturating with a Pasteur pipette. Cells in MEM were plated onto 96- or 24-well plates coated with poly-L-lysine at 15 μg/ml. FUDR (50 μg/ml uridine and 20 μg/ml 5'-fluoro-2-deoxyuridine; Sigma) was added to medium after 4 days in culture to suppress the proliferation of glial cells. Pure neuronal cultures were obtained by maintaining the cells in serum-free medium. Enzymatic assays and immunostaining of Cu/Zn SOD were performed using previously described protocols (15, 18). For immunostaining, cultures were fixed with 10% formaldehyde and permeabilized by incubation in 0.02% Tween 20 and 3% normal goat serum.

Neuronal/Glial Cocultures. Glial feeder layers were obtained by culturing cortical cells from 17-day-old embryos for 12 days in DMEM containing 10% fetal calf serum. Under these conditions, all neurons died and most of the remaining cells were astrocytes, as determined by immunostaining with anti-glial fibrillary acidic protein antibodies (anti-GFAP, Incstar, Stillwater, MN). Neurons dissected from 14-day-old embryos were plated on top of the glial feeder layer and co-cultures were maintained in serum-free medium for 7–9 days before the toxicity assay was performed.

KA Cytotoxicity and $[Ca^{2+}]_i$ Imaging in Cultured Neurons. Cultures were incubated for 16–19 hr in MEM plus 0.05–0.1 mM KA (Tocris Neuramin, Bristol, U.K.). Overall cell injury was determined by counts and morphology. Neurons were identified by immunostaining with anti-neuron-specific enolase (anti-NSE, Incstar). The number of NSE positive cells was determined in at least 15 fields (of 0.25 mm^2 each) per well. Experiments were repeated at least three times using different batches of cultured cells. When indicated, overall cell death was also determined by the decrease in reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

Ca^{+2} concentrations within spinal cord neurons were determined by the ratio of Fura-2 (Molecular Probes) fluorescence due to excitation at 340/380 nm. Neurons were plated on glass coverslips (13-nm diameter) and $[Ca^{2+}]_i$ was determined in 10-day-old cultures as described by Segal and Manor (28). Measurements were made at 10-sec intervals before and following application of drugs by pressure through a glass micropipette.

Apoptosis and DNA Fragmentation. *In situ labeling (TUNEL).* Apoptotic cells in culture were detected by fluorescent labeling of the DNA 3'-ends by terminal deoxynucleotidyl transferase as described by Gavrieli *et al.* (29).

Electron microscopy analysis. Cultured neurons were maintained for 14 days and exposed to 0.1 mM KA, as indicated above. Treated and untreated cultures were fixed, stained, embedded in epoxy resin (Epon), and examined by electron microscopy as described previously (14).

Fluorescent Measurement of Intracellular Glutathione (GSH). Cortical neurons were plated on coverslips as above and incubated for 8 hr either in medium alone or medium plus buthionine sulfoximine (BSO) (25 μM) to attenuate GSH synthesis. H_2O_2 (10^{-5} M) was added for 45 min. Cells were washed twice and incubated for 15 min with 4×10^{-5} M monochlorobimane (mBCl, Molecular Probes). mBCl is freely permeable to cells; once inside the cells, mBCl forms a fluorescent complex with GSH (30). Fluorescence signals were recorded after excitation at 380 nm and mean OD values were calculated. In each experiment, 50–100 neurons per treatment were monitored.

Statistical Analysis. Statistical analysis was performed by multifactor analysis of variance (ANOVA). Where necessary, the variable was first subjected to an angular or logarithmic transformation to stabilize the variances. In cases where the same experiment was repeated on different occasions, all data were analyzed together.

RESULTS

Tg-Cu/Zn SOD Mice and Overexpression of Cu/Zn SOD in Brain and Cultured Neurons. Transgenic mice containing the human Cu/Zn SOD gene were obtained as previously described (15, 31) by microinjecting fertilized eggs with a linear 14.5-kb fragment of human genomic DNA containing the entire Cu/Zn SOD gene, including its regulatory sequences (32). Several transgenic strains expressing various levels of the transgene were bred and analyzed (15–18). The three strains studied here, TgHS 51, TgHS 69, and TgHS 70, contained 4–5 copies of the human Cu/Zn SOD gene in their genome and expressed the transgene as an active enzyme capable of forming the human–mouse heterodimer protein shown in Fig. 1A (13, 15, 31). Outwardly, Tg-Cu/Zn SOD mice used in the present experiments showed no obvious physical abnormalities (16). The enzymatic activity in the brains of Tg-Cu/Zn SOD mice was determined either by the inhibition of nitrite formation (Table 1) or by gel electrophoresis (Fig. 1*A*). Brain extracts of Tg-Cu/Zn SOD mice had 4- to 6-fold higher Cu/Zn SOD activity as compared with nontransgenic control mice with TgHS 51 having the highest and TgHS 70 the lowest increase in Cu/Zn SOD activity (Table 1). The pattern of transgene expression in the brains was further analyzed by immunostaining of horizontal brain sections with anti-human Cu/Zn SOD antibodies. It was found that the general disposition of the human enzyme in the brain sections of the TgHS strains was similar to that of the mouse and staining with

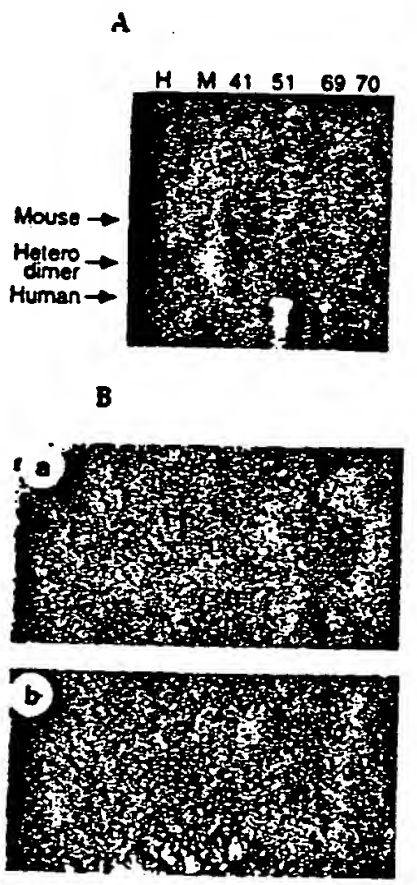

FIG. 1. (*A*) Polyacrylamide gel analysis of Cu/Zn SOD enzymatic activity in brain extracts from control and different lines of Tg-Cu/Zn SOD mice. The positions at which the active human (H) and mouse (M) Cu/Zn SOD enzymes migrate are indicated. (*B*) Immunostaining of cultured neurons with anti-human Cu/Zn SOD antibodies. (*a*) Nontransgenic. (*b*) Cultures derived from TgHS-51 embryos. (×140.)

Table 1. Activity of Cu/Zn SOD in brains of transgenic and control mice

Mouse strain	Cu/Zn SOD
Control	15.6
TgHS 51	98.3 (6.3)
TgHS 69	79.6 (5.1)
TgHS 70	67.1 (4.3)

Cu/Zn SOD specific activity in units per mg of protein in brain extracts was determined. The mean specific activity of extracts from six mice of each strain are given. The values in parentheses represent the fold increase in Cu/Zn SOD activity relative to nontransgenic control mice and were significant at $P < 0.001$.

anti-human Cu/Zn SOD antibodies was not detected in sections from control nontransgenic animals (data not shown).

Cortical Neurons of Tg-Cu/Zn SOD Mice Are More Susceptible to KA-Mediated Excitotoxicity. The majority of cells in cultures established from cortices of 14-day-old mouse embryos were neurons. Enzymatic activity of Cu/Zn SOD in the cultures was assayed as in Fig. 1*A* and yielded comparable results. Fig. 1*B* depicts the expression of immunoreactive human Cu/Zn SOD in the cultured Tg-Cu/Zn SOD neurons. Of the non-neuronal cells present in the culture, the majority were type 1 astrocytes based on their morphology (flat with polygonal shape) and their antigen properties (glial fibrillary acidic protein positive).

The susceptibility of cortical neurons, from transgenic and nontransgenic control mice, to KA-mediated excitotoxicity was investigated. Treatment with KA was selected for two main reasons. First, we noticed that the cortical neurons respond poorly to *N*-methyl-D-aspartate (NMDA) unless maintained in culture for at least 14 days, a phenomenon previously observed by others (32). Second, there are reports documenting the involvement of OFR in KA-mediated neuronal death (see ref. 2 and references therein). Exposure of cultures to 50 or 100 μM KA led to cell death of both Tg-Cu/Zn SOD and nontransgenic neurons; however, significantly more neurons died in the transgenic cultures than in sister nontransgenic cultures (Table 2, rows A and B). The effect of KA was concentration-dependent; more cells died in the presence of 100 μM, and it was partially blocked by adding the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxalin-2,3-dione (CNQX) (Table 2, row C).

The increased susceptibility of transgenic neurons to KA was further investigated in cortical cultures that were made astroglia-free by growing them in synthetic medium. Under these conditions, although the KA-induced mortality of transgenic neurons was still higher than control, the difference was smaller particularly in the presence of 50 μM KA (Table 2, row D). These results suggest that while Tg-Cu/Zn SOD neurons alone were more susceptible to KA-mediated cell death, the astroglia contributed to the phenomenon as well. To further investigate this observation, cocultures of astroglia and neurons, from either transgenic or control mice, were prepared. Cortical cells were obtained from 17-day-old embryos and grown under conditions that eliminate neurons. As judged by immunostaining of cultures with anti-glial fibrillary acidic protein antibodies, the glial feeder layer was composed mostly of astrocytes. Cocultured neurons were dissected from 14-day-old embryos and plated in synthetic medium on top of the astroglial layer. For each experiment, the same batch of neurons was seeded on top of either transgenic or nontransgenic astroglia and the cocultures were grown in synthetic medium for 7–8 days before the addition of KA. More neurons (either transgenic or nontransgenic) survived the KA treatment when seeded on top of nontransgenic astroglia than when seeded on transgenic astroglia (Table 2, row E). These data indicate that the transgenic astroglia protected neurons against KA-mediated cell death to a lesser extent than did the control nontransgenic astroglia and thereby contributed to the higher sensitivity of the transgenic cultures.

Table 2. KA-mediated cell death in cultured cortical neurons Tg-Cu/Zn SOD and nontransgenic control mice

	Mice and culture	Treatment	% cell d
A			
	Nontransgenic	50 μM KA	11 ± 3
	TgHS 51	50 μM KA	55 ± 5*
	TgHS 69	50 μM KA	40 ± 8*
B			
	Nontransgenic	100 μM KA	47 ± 6
	TgHS 51	100 μM KA	66 ± 4*
C			
	TgHS 51	100 μM KA+CNQX	36 ± 3*
D			
	Nontransgenic/SM	50 μM KA	16 ± 5
	TgHS 69/SM	50 μM KA	33 ± 7*
	TgHS 70/SM	50 μM KA	31 ± 4*
E			
	Cont. Neu/Cont. Glia	50 MM KA	28 ± 3
	Cont. Neu/Tg. Glia	50 μM KA	40 ± 2**
	Tg. Neu/Cont. Glia	50 μM KA	32 ± 4
	Tg. Neu/Tg. Glia	50 μM KA	51 ± 2**

Cortical neurons were maintained in culture for 12 days as described. Viability of neurons was determined, following 16 hr incubation with KA, by the tetrazolium reduction assay (MTT) or by the number of immunostained positive cells as described. Row C, cells were preincubated for 1 hr with 10 μM of the indicated antagonist after which KA was added for a further incubation of 16 hr. Row D, cells were grown in serum free synthetic medium (SM), as described. Values are the average ± SEM of 3-5 experiments. Row E, cocultures were obtained as described. Cont., not transgenic; Tg., transgenic. Values are the mean ± SEM of 3-4 experiments. *, $P < 0.05$; **, $P < 0.01$, as determined by ANOVA.

Cultured Tg-Cu/Zn SOD Spinal Cord Neurons Are Similarly Susceptible To Excitotoxicity. The findings that cortical neurons of Tg-Cu/Zn SOD mice are more susceptible than control neurons to KA-mediated cell death prompted us to examine the response of spinal cord neurons to KA and NMDA. Spinal cord cultures give rise to a mixture of interneurons, dorsal root ganglia, and large motorneurons. The overall sensitivity of spinal cord neurons to KA was higher than that of the cortical neurons, for example, at 50 μM KA the mortality of nontransgenic spinal cord neurons was four times higher than that of nontransgenic cortical neurons (compare Table 2, row A, and Table 3, row A). The transgenic spinal cord cultures, like the cortical neurons analyzed above, were more susceptible to KA than nontransgenics (Table 3, row A) and this effect was attenuated by CNQX (data not shown). Further examination revealed a much higher sensitivity of the large motorneurons population to KA. Addition of 50 μM KA completely eliminated the large motorneurons from the spinal cord cultures (Table 3, row B). As noted above, the cortical neurons respond poorly to NMDA unless maintained in culture for at least 14 days. Spinal cord neurons, on the other hand, respond to NMDA much earlier. When exposed to NMDA for 30 min, more cells died in transgenic spinal cord cultures than in nontransgenic control cultures (Table 3, row C). This indicated that NMDA receptors as well are involved in the increased sensitivity of transgenic cultures to excitotoxicity. Taken together, the results from the KA and NMDA treatments and the coculture studies demonstrate that the cultured Tg-Cu/Zn SOD neurons, cortical, and spinal cord were more susceptible to KA-mediated excitotoxicity and that both NMDA and non-NMDA receptor subtypes were involved.

The KA-Evoked Elevation In Intracellular $[Ca^{2+}]_i$ Is Increased in Tg-Cu/Zn SOD Neurons. Excitotoxic cell death is usually accompanied by the infiltration of excessive amounts of Ca^{2+} into the cell, either through NMDA receptor-linked

Table 3. KA- and NMDA-medi[illegible]cell death in cultured spinal cord neurons of Tg-Cu/Zn SOD and nontransgenic mice

Mice and culture	Treatment	% cell death
A		
Nontransgenic SCN	50 μM KA	48 ± 9
TgHS 51 SCN	50 μM KA	62 ± 10
Nontransgenic SCN	25 μM KA	24 ± 8
TgHS 51 SCN	25 μM KA	40 ± 7*
B		
Nontransgenic LMN	50 μM KA	92 ± 3
TgHS 51 LMN	25 μM KA	98 ± 3
C		
Nontransgenic	50 μM NMDA	35 ± 5
TgHS 51 SCN	50 μM NMDA	58 ± 9**
TgHS 69 SCN	50 μM NMDA	55 ± 4**
Nontransgenic SCN	50 μM KA minus Ca^{2+}	BDL
TgHS 51 SCN	50 μM KA minus Ca^{2+}	BDL

Spinal cord neurons (SCN) and large motor neurons (LMN) were obtained from 13-day-old embryos as described. Cultures were incubated with KA or NMDA as described. Viability of neurons was determined as in Table 2. Values are the average ± SEM of 3-5 experiments. *, $P < 0.01$; **, $P < 0.05$, as determined by ANOVA. BDL, below detection level.

calcium channels or through voltage-gated calcium channels. [illegible]e exposed spinal cord and cortical neurons to 50 μM KA in Ca^{2+}-free medium to further characterize the KA-induced toxicity and found that, under these conditions, cell death was completely dependent on the presence of calcium (Table 3, row D). This indicated that calcium influx was necessary for the observed neuronal damage. The changes in the level of $[Ca^{2+}]_i$ within the cell after brief glutamate pulses were then monitored using Fura-2 calcium imaging. Significant differences in Ca^{+2} concentrations were found between Tg-Cu/Zn [illegible]OD and nontransgenic neurons in the initial response (peak/[illegible]asal) and in the recovery phase (Table 4). Immediately after [illegible] glutamate pulse, $[Ca^{2+}]_i$ went up to a higher value in Tg-neurons than in control neurons and stayed higher after 30–40 sec. The data indicate that calcium-homeostasis was altered in the Tg-Cu/Zn SOD neurons. In particular, the prolonged recovery phase is meaningful since the duration of time for which neurons are exposed to elevated $[Ca^{2+}]_i$ is an important contributor to Ca^{2+}-mediated cytotoxicity.

The KA-Induced Neuronal Cell Death Is Apoptotic. The [illegible]uestion of whether exposure to KA induces apoptosis in our [illegible]ltured neurons was addressed using biochemical and ultra[illegible]tructural analyses. Internucleosomal cleavage of DNA is evident in almost all apoptotic cells (for review, see ref. 34). We used the "TUNEL"-nick-end labeling procedure (transfer of biotinylated nucleotides to the 3'-OH ends) of Gavrieli *et al.* (29) to record *in situ* DNA fragmentation. A distinct pattern of nuclear staining in KA-treated cultures was detected (Fig. 2*I*). This staining was eliminated by the addition of 100 μM aurintricarboxylic acid (ATA), known to inhibit the Ca^{2+}-dependent endonuclease responsible for apoptotic DNA cleavage (27).

Table 4. Alteration of $[Ca^{2+}]_i$ peak response and recovery in Tg-Cu/Zn SOD neurons

Culture	Change in response*	Change in recovery†
Nontransgenic	5.8	0.38
TgHS 51	9.0‡	0.65‡
TgHS 69	8.4‡	0.57‡

$[Ca^{2+}]_i$ was determined as described.
*Ratio of $[Ca^{2+}]_i$ peak/basal. Basal values were 50 ± 9 nM and peaks values ranged from 250 ± 4 to 370 ± 7 nM.
†Ratio of $[Ca^{2+}]_i$ at 30-40 sec/peak. Values at 30-40 sec ranged between 95 ± 5 to 235 ± 7 nM. Values are the mean ± SEM of 70-160 cells that were analyzed in nine separate experiments.
‡Significantly different from values of nontransgenic control cultures ($P < 0.05$) as described.

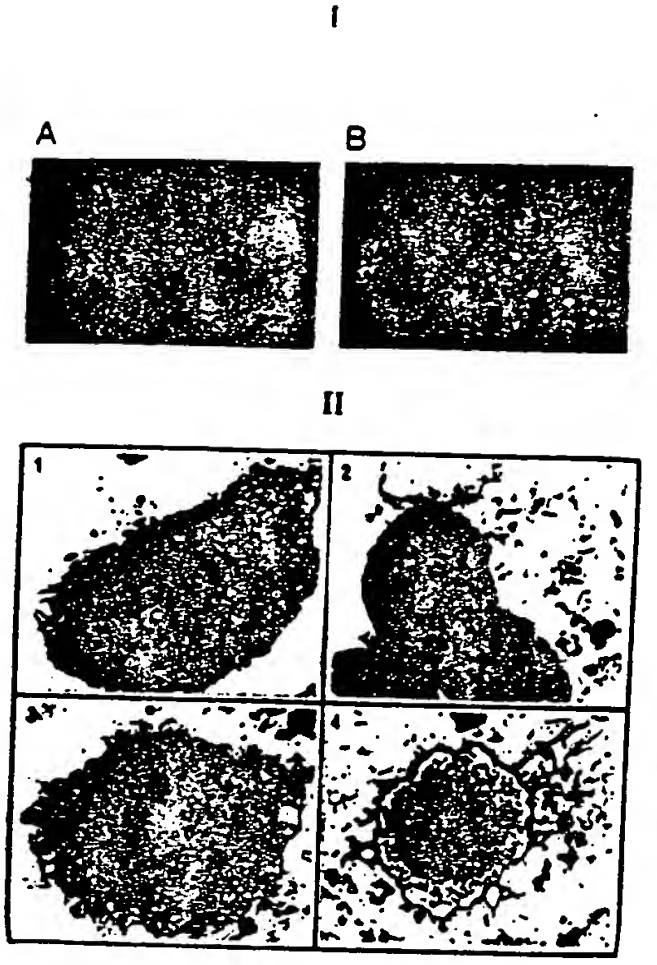

FIG. 2. Documentation of KA-mediated apoptosis of cultured neurons. (*I*) *In situ* DNA fragmentation assay using terminal transferase mediated dUTP nick-end labeling (TUNEL) method. Spinal cord neurons untreated (*A*) or treated with 0.1 mM KA (*B*). Fluorescence signals were obtained by excitation of fluorescein isothiocyanate at 485 nm. (*II*) Transmission electron micrographs showing typical stages of neurons undergoing KA-mediated apoptosis. (*1*) Untreated neuron. (*2*) Neuron at early stages of apoptosis. (*3* and *4*) Advanced apoptosis: patches of condensed chromatin and abundant vacuoles. (×56,700.)

Ultrastructural features of apoptotic cells are readily visualized by electron microscopy. In untreated cultures, the majority of cells displayed normal ultrastructures: well-preserved organelles, abundant cytoplasm, and dispersed chromatin in the nucleus (Fig. 2*II1*), whereas in the KA-treated cultures numerous cells exhibited early nuclear changes, patches of condensed chromatin, and abundant vacuolization in the cytoplasm (Fig. 2 *II3* and *II4*). We concluded that under the experimental conditions described above, KA induced Ca^{2+}-dependent apoptotic cell death in the cultured neurons and that Tg-Cu/Zn SOD neurons were more severely affected.

Reduced Levels of Intracellular Glutathione (GSH) in Tg-Cu/Zn SOD Neurons. Constitutive overexpression of Cu/Zn SOD may lead to chronic oxidative stress which could explain the higher sensitivity of Tg-Cu/Zn SOD cultures to excitotoxicity. The tripeptide GSH is present in cells at millimolar concentrations. Under normal conditions, most of the GSH is maintained in its reduced form by glutathione reductase. GSH reacts readily with reactive oxygen species, thereby providing protection against oxidative injury. Continual production of reactive oxygen species, such as H_2O_2, •OH, and lipid peroxides, leads to accumulation of oxidized glutathione (GSSG), and a concomitant reduction in the level of GSH. Therefore, the intracellular level of GSH provides a relevant and accurate measure of the [illegible]xidative state of the cell. We used mBCl to directly monit[illegible]r GSH levels within the cells.

Table 5. Analysis of intracellular levels of GSH in cultured neurons

Treatment	% positive cells: Nontransgenic		Transgenic SOD	
None				
Exp. 1	23	19 ± 14	36	33 ± 5
Exp. 2	16		29	
+ BSO				
Exp. 1	56	41 ± 1	89	74 ± 12
Exp. 2	38		74	
Exp. 3	30		60	
$+H_2O_2$				
Exp. 1	55	41 ± 1	92	75 ± 12
Exp. 2	35		64	
Exp. 3	44		69	

Cortical neurons were plated on glass coverslips and assayed as described. Cultures of both TgHS 51 and TgHS 69 were examined. For each treatment, 50-100 neurons were recorded and values depict the % positive cells, defined as those with fluorescence intensity < 20 OD Numbers centered on braces are the average ± SD of three experiments and they were significantly different from nontransgenic ($P < 0.05$, see *Materials and Methods*).

mBCl reacts with GSH in a reaction catalyzed by glutathione *S*-transferase, yielding fluorescent mBCl (30). Analysis of fluorescence intensity revealed that, compared with control, transgenic cultures contained higher proportions of positive cells, i.e., neurons with low levels of GSH (Table 5). The phenomenon was enhanced in the cultures preincubated with low concentrations of buthionine sulfoximine (BSO), which attenuates the synthesis of GSH. BSO is a specific inhibitor of γ-glutamyl-cysteine synthetase and can therefore be used to decrease the intracellular concentrations of reduced glutathione. Similarly, the effect of H_2O_2 causing a rapid depletion of cellular GSH was more pronounced in the transgenic neurons (Table 5). The addition of 2 mM diethyl maleate, which reacts with the glutathione SH group, completely abolished the mBCl fluorescence. The results demonstrate a significant reduction of GSH levels in Tg-Cu/Zn SOD neurons and support the premise that increased Cu/Zn SOD activity creates chronic oxidative stress that may explain the higher susceptibility of the transgenic neurons to excitotoxicity.

DISCUSSION

Cu/Zn SOD is a key enzyme in the metabolism of OFR. The human gene encoding Cu/Zn SOD resides on chromosome 21 and is overexpressed in DS patients. Recently, alterations in expression and enzymatic properties of Cu/Zn SOD were implicated in certain cases of familial ALS (for review, see ref. 25). This provided sound genetic evidence that altered metabolism of OFR could be involved in neurodegenerative diseases. Moreover, transgenic mice overexpressing the Cu/Zn SOD gene bearing ALS-linked mutations develop motor neuron disease with many clinical and pathological features of human ALS (see ref. 25 and references therein), highlighting the possibility of oxygen radical involvement in motor neuron death.

Several laboratories have previously found that constitutive overexpression of wild-type Cu/Zn SOD or MnSOD in cells and animals caused highly specific oxidative injuries (13–22, 29, 35–37). In extending these findings, we have now analyzed the effect of Cu/Zn SOD gene-dosage on survival of cortical and spinal cord neurons.

We dem nstrated that cultured neurons from three distinct lines of Tg-Cu/Zn SOD mice were more vulnerable to KA-mediated excitotoxicity than neurons from control nontransgenic mice. The data are consistent with the possibility that the increased susceptibility to KA is due to a chronic prooxidant state in the transgenic neurons. Destabilization of calcium homeostasis in the neurons. Destabilization of calcium homeostasis has been considered to be a major mechanism of excitotoxic cell death (for review, see ref. 38). Under normal physiological conditions, there is an inherent equilibrium between oxidant and antioxidant mechanisms, but even though the physiological activity of Cu/Zn SOD is associated to some degree with generation of hydroxyl radicals (10–12). When Cu/Zn SOD activity was increased, the balance was altered, creating indigenous oxidative stress in the transgenic cultures as indicated by the marked reduction of intracellular GSH. Exposure to H_2O_2 caused a similar depletion of intracellular GSH, indicating that peroxides and downstream products are the damaging agents. Importantly, the H_2O_2-dependent depletion of GSH was significantly greater in the transgenic neurons than in control nontransgenic cells. As has been noted before, increased H_2O_2 may lead to production of •OH radicals through Fenton's reaction and then to cell death via apoptosis (7, 8, 39). The existence of indigenous oxidative stress within the cell depends, among other factors, on the ratio between the rate of superoxide production and amount of Cu/Zn SOD present (40). Therefore, lesions caused by various exogenous insults could either be ameliorated or worsened, as was indeed the case in transfected-Cu/Zn SOD cells (13, 19, 20, 41) and Tg-Cu/Zn SOD mice (42–49).

Increased concentrations of intracellular $[Ca^{2+}]_i$ is a major consequence in cells exposed to KA or glutamate and the rate of Ca^{2+} removal from the cytoplasm is an important factor in the cell's ability to survive an excitotoxic challenge (for review, see ref. 38). Using Fura-2 imaging, we showed that glutamate evoked a sustained and greater increase in internal $[Ca^{2+}]_i$ in transgenic neurons as compared with control cultures. The latter responded with an initial rise that rapidly returned to near baseline, whereas transgenic neurons exhibited a prolonged recovery phase and their internal $[Ca^{2+}]_i$ remained high for a longer period. These data indicate that Tg-Cu/Zn SOD neurons are less efficient at extruding Ca^{2+}, which may explain their relatively higher sensitivity to KA even in astroglia-free cultures. The biochemical basis of this handicap of the transgenic neurons might be an altered activity of the Na^+/Ca^{2+} exchanger (38). This exchanger plays a significant role in extruding intraneuronal Ca^{2+}; when this action is inhibited, glutamate neurotoxicity is potentiated. Activity of the Na^+/Ca^{2+} exchanger, as that of the glutamate transporter, is dependent upon maintenance of the sodium gradient by Na^+/K^+ ATPase (38). In PC12 neuronal cells, overexpression of Cu/Zn SOD caused oxidative stress, leading to a damage in the chromaffin granule H^+ ATPase, thus reducing uptake of neurotransmitters into the vesicles (14). A similar defect, affecting the uptake of blood serotonin, was also identified in Tg-Cu/Zn SOD platelets (17). It is conceivable that the Na^+/K^+ ATPase of the transgenic neurons and astroglia were inflicted in the same manner, leading to the observed alteration in calcium homeostasis.

Cell death is believed to occur through one of two well-distinguished pathways, apoptosis or necrosis, that differ from each other morphologically and biochemically (for review, see ref. 34). Ultrastructural analysis and *in situ* labeling with terminal transferase of KA-treated neurons revealed that, in our cultures, KA-induced cell death is apoptotic. Aurintricarboxylic acid, a general nuclease inhibitor known to prevent apoptosis in neurons and PC12 cells, suppressed KA-induced terminal labeling and abrogated neuronal death. These results are in agreement with reports implicating KA-induced toxicity and oxidative stress in neuronal apoptosis.

DS patients are inflicted by an early onset of AD and virtually all DS patients develop the AD pathology during the fourth decade of their lives (for review, see ref. 23). One of the neuropathological hallmarks of AD is the accumulation of

...onally found in brains of DS patients less than 3 years old ...51). The gene encoding the β-amyloid precursor protein ...PP) resides on chromosome 21 and is overexpressed in DS ... ons (52). In addition, certain forms of familial AD are ...ciated with mutations in the APP gene that lead to ...rproduction of Aβ (see ref. 50 and references therein). This ...pports the hypothesis that Aβ accumulation may contribute ...o the progression of the disease. The Aβ peptide is directly toxic to neurons through its tendency to form insoluble aggregates that potentiate glutamate excitotoxicity and disrupt Ca^{++} homeostasis (53, 54). The molecular details of the latter processes are not completely known but involve increased accumulation of H_2O_2 related peroxides within the cells leading to oxidative damage and cell death (see ref. 39 and ...ferences therein). It was recently shown that cultured cortical ...rons from DS fetuses undergo apoptosis due to increased ...neration of OFR and that cell death can be prevented by free-radical scavengers or catalase but not SOD (24). As presented here and discussed above, constitutive overexpression of Cu/Zn SOD in cells and transgenic mice causes long-term oxidative stress, as marked by increased lipid peroxidation and specific oxidative damage to membrane associated enzymes. The Tg-Cu/Zn SOD mice exhibited several phenotypic features found in DS patients including abnormalities in the neuromuscular junctions and a decrease in blood ...otonin levels caused by a defect in the platelet granule ...nsport system. The data presented here show that neurons of Tg-Cu/Zn SOD mice are more susceptible to degeneration when subjected to added insults such as treatment with KA. Given the fact that both Aβ and Cu/Zn SOD are elevated in DS patients, who usually develop AD pathology early in life, it will be interesting to investigate the toxic effect of Aβ on the already imperiled Tg-Cu/Zn SOD neurons. This may contribute to our understanding of the genetic predisposition of DS ...tients to the early onset of AD pathology.

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